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TECHNICAL NOTE

Detection of rifampicin resistance in *Mycobacterium tuberculosis* isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance

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SUMMARY

The line probe assay (LiPA), a rapid molecular method for detecting rifampicin resistance (RMP^r) in *Mycobacterium tuberculosis*, correctly identified all 145 rifampicin-sensitive (RMP^s) and 262 (98.5%) of 266 RMP^r strains among 411 isolates collected from diverse countries. If used as a marker of multidrug-resistant tuberculosis (MDR-TB), detection of RMP^r by LiPA would have detected 236 of the 240 MDR strains in this study but would have wrongly suggested the presence of MDR in 26 RMP-monoresistant isolates (sensitivity 98.3%, specificity 84.8%). Hence, the reliability of using LiPA (or any other rapid RMP^r-detection method) as a surrogate marker of MDR-TB largely depends on the prevalence of RMP-monoresistance in the study population. This approach must therefore be validated in each local situation.

KEY WORDS: tuberculosis; rifampicin; multidrug resistance

MULTIDRUG-RESISTANT TUBERCULOSIS (MDR-TB), defined as resistance to at least isoniazid (INH) and rifampicin (RMP), is an increasing threat to effective tuberculosis (TB) control in both industrialised and developing countries.[1] Patients infected with MDR strains are less likely to be cured, and second-line treatments are more toxic and expensive.[2] Successful treatment of MDR-TB relies on prompt laboratory detection of drug resistance.[3,4] Rapid molecular detection of rifampicin resistance (RMP^r) is now possible because the genetic basis for this resistance has been largely elucidated [reviewed in reference 5]. Over 90% of RMP^r strains have mutations in the 81-bp core region of the *rpoB* gene, which encodes the β-subunit of the RNA polymerase. Various molecular techniques, including DNA sequencing, heteroduplex analysis, polymerase chain reaction (PCR) single-stranded conformational polymorphism (PCR-SSCP) and line probe assay (LiPA) have been used as a rapid screening method for these mutations.[6–8]

Two recent evaluations of the commercial LiPA have proposed that detection of RMP^r by this rapid molecular method may be a useful surrogate marker of MDR-TB.[8,9] Our laboratory has published an evaluation of the LiPA assay based on the testing of 203 RMP^r and 61 RMP^s clinical isolates from diverse geographic regions.[10] Since that study, we have tested additional clinical isolates (63 RMP^r and 84 RMP^s) by LiPA and analysed the cumulative data to determine whether detection of RMP^r by this assay could be an early indicator of MDR-TB.

A total of 1562 *Mycobacterium tuberculosis* complex isolates collected for patient care or during drug susceptibility surveys in various countries were referred to our laboratory as part of quality assurance programs (Table 1).[11] Antibiotic susceptibility tests (AST) were performed on Löwenstein-Jensen (L-J) medium by the proportion method.[12] The recommended critical concentrations of RMP and INH (i.e., 40 and 0.2 μg/ml, respectively) for performing ASTs on L-J medium were used.[12]

The commercial LiPA (INNO-LiPA Rif.TB, Innogenetics NV, Zwjindrecht, Belgium) was performed according to the manufacturer's instructions on a subset of 411 isolates (Table 1).[6] This subset contained 145 RMP^s and 266 (49.5%) of the RMP^r isolates (as detected by AST), including all 26 RMP-monoresistant strains. In the LiPA assay, the central region of the *rpoB* gene is amplified and mutations detected by reverse hybridization.[6] Ten specific oligonucleotide probes (one specific for *M. tuberculosis* complex, five overlapping wild-type probes that cover the hypervariable core region of the *rpoB* gene, and four mutation-specific probes) are immobilised at known locations on a membrane strip and hybridised under stringent conditions with the biotin-labelled

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Table 1 Geographic origins and drug susceptibility patterns of *M. tuberculosis* strains by the proportion method and the INNO-LiPA Rif.TB tests

Origin (*n*)	Proportion method				Isolates resistant by LiPA
	INH^r/RMP^r	INH^r/RMP^s	INH^s/RMP^r	INH^s/RMP^s	
Asia (462)*	165	125	3	19	37/38
North Africa (78)†	35	10	1	32	22/22
Sub-Saharan Africa (176)‡	60	22	2	92	54/56
Western Europe (251)§	37	12	7	195	26/26
Eastern Europe (258)¶	97	65	3	93	35/35
Central Europe (267)**	115	60	6	86	56/57
South America (21)††	4	4	3	10	7/7
Control strains (49)‡‡	24	12	1	12	25/25
Total (1562)	537	310	26	689	262/266

* Bangladesh, Kazakhstan, Pakistan.
† Algeria, Egypt, Libya, Tunisia.
‡ Benin, Burkina-Faso, Burundi, Congo, Democratic Republic of Congo, Guinea, Senegal, Rwanda.
§ Belgium, France, Luxembourg.
¶ Abkhasia, Azerbaidjan, Georgia, Ossetia (south).
** Romania, Russia (Siberia).
†† Colombia, Honduras, Peru.
‡‡ Strains from WHO quality control study for drug resistance.[11]
LiPA = line probe assay; INH^r = strains resistant to INH; RMP^r = resistant to RMP; RMP^s = susceptible to RMP; INH^s = susceptible to INH.

PCR product. The hybrids formed are subsequently detected colorimetrically.

In vitro susceptibility testing of the 1562 samples found 563 RMP^r isolates; of these, 537 (95.4%) were also INH^r and thus MDR (Table 1). Only 26 (4.6%) were INH^s (i.e., RMP-monoresistant). Importantly, MDR-TB (INH^r/RMP^r) and RMP monoresistance (INH^s/RMP^r) were detected in all the geographic regions analysed (Table 1). Although it contained predominantly AST survey samples, this cohort cannot be considered representative of these various regions because clinical samples from referral centres were included and some areas were represented by only a handful of isolates. Nonetheless, this cohort provided a convenient subset of samples from diverse geographic regions on which to evaluate the LiPA test.

The results of the INNO-LiPA Rif.TB test on the subset of 411 isolates are presented in Table 2. All 145 (100%) RMP^s isolates and 262/266 (98.5%) of RMP^r isolates were correctly identified by the INNO-LiPA Rif.TB test. Four isolates were RMP^r by the proportion method but RMP^s by the INNO-LiPA Rif.TB test. Sequencing of the central region of the *rpoB* gene showed no mutation in these four isolates (data not shown). These 'false-negative' results may result from mutations in other regions of the *rpoB* gene, as found in *Escherichia coli*,[13] or different resistance mechanisms such as degradation of RMP by decomposition or glycosylation as described in *Nocardia* and certain rapidly growing mycobacteria such as *M. smegmatis*.[14]

Table 2 Comparison of results for rifampicin susceptibility obtained by the proportion method and LiPA for 411 *Mycobacterium tuberculosis* isolates

		Proportion method*		
		RMP^r/INH^r	RMP^r/INH^s	RMP^s
LiPA	RMP^r	236	26	
	RMP^s	4†		145

* Results of the INNO-LiPA Rif.TB assay are tabulated for 240 isolates demonstrating resistance to rifampicin (RMP^r) and isoniazid (INH^r), 26 isolates with rifampicin monoresistance (RMP^r/INH^s), and 145 rifampicin-susceptible strains (RMP^s).
† Two of these strains were isolated from TB patients from Rwanda, one from Romania and one from Bangladesh.
LiPA = line probe assay; RMP^r = strains resistant to RMP; INH^r = resistant to INH; INH^s = susceptible to INH; RMP^s = susceptible to RMP.

The majority of rifampicin resistance in *M. tuberculosis* has been attributed to just two amino acid substitutions, *His526Tyr* and *Ser531Leu*, in the RNA polymerase β-subunit.[5] Unfortunately, RMP^r isolates from developing countries have not been extensively studied, and other mutations may predominate in specific geographic areas, thereby invalidating LiPA and other molecular detection methods of RMP^r detection. For example, Kim et al. reported a novel substitution, *Gly507Asp*, among RMP^r strains from Korea.[7] None of the five LiPA evaluations by other researchers studied more than 75 samples,[8,9,15–17] and all but one used samples collected in only one city or industrialised country (e.g., New York City, Greece, Spain, or the United Kingdom). By using a large sample cohort from diverse regions, this study has shown that LiPA is robust and accurately detects 98.5% (95% confidence interval 96.3–99.7) of RMP^r strains from seven different geographic regions. This performance is consistent with previous evaluations in which LiPA has detected 90.2%–100% of isolates defined as RMP^r by in vitro susceptibility tests.[8–10,15–17]

This study also investigated the suggestion that rapid RMP^r detection by LiPA may be used as an initial indicator of MDR-TB.[8,9] Unfortunately, false-positive and false-negative results confound the use of LiPA (and other molecular techniques) for this purpose. In this study, LiPA detected 236 (98.3%) of the

240 MDR-TB isolates tested (Table 2). However, the four RMP^r strains in this study that were not detected by LiPA were also INH^r, and therefore represented false-negative results in the detection of MDR-TB. LiPA has a demonstrated sensitivity of over 90% in detecting RMP^r isolates,[8–10,15–17] so false-negative results in the detection of MDR-TB should be expected but limited (as in this study).

False-positive results actually represent a greater problem for this strategy. Twenty-six strains demonstrating RMP monoresistance would have been incorrectly identified as MDR-TB in this study. The extent of these false-positive results will depend on the prevalence of RMP monoresistance in the study population. Our in vitro susceptibility testing detected RMP monoresistance in all regions (Table 1), and the largest AST survey has reported primary rifampicin monoresistance from 19 countries, with the highest rate being 6.9% in Thailand and the Dominican Republic.[1] A survey of TB isolates collected in the United States between 1993 and 1996 also found increasing rates of RMP monoresistance, where 2.6% of HIV-positive cases had RMP monoresistance compared with only 0.2% of HIV-negative cases.[18] Hence, the main weakness of using rapid detection of RMP^r by INNO-LiPA Rif.TB test as an early indicator of MDR would appear to be the false detection of RMP-monoresistant isolates as MDR. As a surrogate marker of MDR-TB, rapid detection of RMP^r by other molecular techniques faces the same inherent weakness. However, early detection of RMP-monoresistant isolates as 'drug-resistant' is still important, because rifampicin resistance precludes treatment using standard short-course chemotherapy.

False-positive results in the early detection of MDR-TB could also occur if LiPA lacked specificity in discriminating RMP^s strains. LiPA correctly identified all 145 RMP^s in this study, and previous evaluations have also reported 100% specificity.[8–10,15–17] However, Kim et al. have recognised false-positive results with the PCR-SSCP test due to a silent substitution and a deletion mutation in the *rpoB* gene.[7] The LiPA assay could be similarly affected.

Numerous molecular methods (e.g., DNA sequencing, heteroduplex analysis, PCR-SSCP) have been proposed for the rapid detection of RMP^r.[6–8] In contrast to these alternatives, LiPA is a validated commercial assay that requires limited equipment (i.e., a PCR thermocycler and a thermostatic shaking water bath for the hybridisation step) and minimal technical expertise in molecular biology. When performed on a liquid culture or a loop-full of colonies harvested from solid medium, the assay can be completed within one working day.[6] LiPA can also be performed directly on sputum specimens, but the additional sample-preparation step extends the test time to 2 days. The reagent cost for the LiPA assay is about US$16 per test.

In conclusion, this study has detected MDR-TB and RMP monoresistance in all geographic regions analysed, using a large cohort of samples from diverse countries to validate the INNO-LiPA Rif.TB test as a robust, rapid method of detecting RMP^r. Furthermore, when rapid RMP^r detection is used as a surrogate marker, LiPA correctly detected 98.3% of MDR-TB strains tested. However, the utility of this strategy depends on the sensitivity and specificity of LiPA, and the prevalence of RMP monoresistance in the study population. Unfortunately, the 1562 samples in this study are not a representative cohort, and only 266 (49.5%) of the RMP^r strains were subjected to LiPA, including only seven RMP^r strains from South America. Hence, the performance of LiPA and its usefulness as an early indicator of MDR-TB in any specific country must still be validated and confirmed by AST.

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RÉSUMÉ

Le test d'hybridation inverse de sondes en lignes (LiPA), une méthode moléculaire rapide de détection de la résistance à la rifampicine (RMP^r) chez *Mycobacterium tuberculosis*, a identifié correctement tous les 145 souches sensibles à la rifampicine (RMP^s) ainsi que 262 des 266 souches RMP^r (98,5%) parmi 411 isolats provenant de différents pays. S'il avait été utilisé comme marqueur d'une tuberculose multirésistante (MDR-TB), la détection des RMP^r par la méthode LiPA aurait détecté 236 des 240 souches MDR de cette étude mais aurait aussi suggéré erronément la présence de MDR dans 26 isolats monorésistants à la RMP (sensibilité 98,3%, spécificité 84,8%). Dans ces conditions, la fiabilité de l'utilisation du LiPA (ou de n'importe quelle autre méthode rapide de détection de RMP^r) comme marqueur alternatif de MDR-TB dépend dans une large mesure de la prévalence de la monorésistance à la RMP dans la population étudiée. Cette approche doit dès lors être validée dans chaque situation locale.

RESUMEN

El test de prueba de sondas en líneas (line probe assay—LiPA), un método molecular rápido para detectar la resistencia a la rifampicina (RMP^r) de *Mycobacterium tuberculosis*, identificó correctamente a 145 cepas sensibles a la rifampicina (RMP^s) y 262 (98,5%) de 266 RMP^r entre 411 cepas obtenidas de diferentes países. Si se utiliza como un marcador de tuberculosis multirresistente (MDR-TB), la detección de RMP^r por el LiPA hubiera detectado 236 de las 240 cepas MDR en este estudio, pero hubiera sugerido erróneamente la presencia de MDR en 26 cepas mono-resistentes (sensibilidad 98,3%, especificidad 84,8%). Por lo tanto, la confiabilidad de utilizar LiPA (o cualquier otro método rápido de detección de RMP^r) como un marcador alternativo de MDR-TB depende sobre todo de la prevalencia de la mono-resistencia a la RMP en la población estudiada. Este enfoque debe ser validado en cada situación local.